Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770

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Edited by Michael J. Welsh, University of Iowa, Iowa City, IA, and approved September 4, 2009 (received for review May 4, 2009)

Cystic fibrosis (CF) is a fatal genetic disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), a protein kinase A (PKA)-activated epithelial anion channel involved in salt and fluid transport in multiple organs, including the lung. Most CF mutations either reduce the number of CFTR channels at the cell surface (e.g., synthesis or processing mutations) or impair channel function (e.g., gating or conductance mutations) or both. There are currently no approved therapies that target CFTR. Here we describe the in vitro pharmacology of VX-770, an orally bioavailable CFTR potentiator in clinical development for the treatment of CF. In recombinant cells VX-770 increased CFTR channel open probability (P_o) in both the F508del processing mutation and the G551D gating mutation. VX-770 also increased Cl^- secretion in cultured human CF bronchial epithelia (HBE) carrying the G551D gating mutation on one allele and the F508del processing mutation on the other allele by ≈10-fold, to ≈50% of that observed in HBE isolated from individuals without CF. Furthermore, VX-770 reduced excessive Na^+ and fluid absorption to prevent dehydration of the apical surface and increased cilia beating in these epithelial cultures. These results support the hypothesis that pharmacological agents that restore or increase CFTR function can rescue epithelial cell function in human CF airway.

cystic fibrosis transmembrane conductance regulator (CFTR) | epithelial ion transport | epithelial sodium channel (ENaC) | human bronchial epithelium (HBE) culture

Drugs that repair the function of proteins that are defective because of gene mutations offer hope for the treatment of genetic diseases such as cystic fibrosis (CF). CF is a fatal genetic disease caused by mutations on both alleles in the gene encoding CFTR (1, 2), a protein kinase A (PKA)-activated epithelial Cl^- and HCO_3^- selective ion channel involved in salt and fluid transport in multiple organs (3–7). Although CF has many clinical manifestations, lung disease is the primary cause of morbidity and mortality (8). One hypothesis to explain the link between defective CFTR and CF lung pathogenesis is that loss of CFTR-mediated Cl^- secretion causes airway surface dehydration because of both a decrease in CFTR-mediated Cl^- and fluid secretion and a secondary increase in epithelial Na^+ channel (ENaC)–mediated Na^+ and fluid absorption (9–11). The resulting dehydration of the airway surface is believed to contribute to the deleterious cascade of mucus accumulation, infection, inflammation and destruction that characterizes CF lung disease (12). Current therapies to treat CF lung disease, including mucolytics, antibiotics, and anti-inflammatory agents, treat downstream disease processes that are secondary to the loss of CFTR function. An alternative therapeutic strategy to treat CF is to increase CFTR function by gene therapy or drugs, with the expectation that this would stop the pleiotropic consequences of *CFTR* mutations and would reduce the severity or slow the progression of the disease.

A number of therapeutic discovery and development efforts are underway to develop drugs that repair the underlying molecular defects in CFTR caused by different *CFTR* mutations (13, 14). The most common *CFTR* mutation, F508del, accounts for ≈70% of all *CFTR* alleles in patients with CF (15). The F508del mutation impairs the intracellular processing and subsequent delivery of CFTR to the cell surface resulting in the loss of CFTR-mediated Cl^- and HCO_3^- secretion (13). Pharmacological agents that increase the cellular processing and delivery of CFTR proteins, such as F508del CFTR, to the cell surface to increase the flow of ions are called CFTR correctors (14, 16, 17). Although less common, *CFTR* mutations that primarily impair the ability of CFTR at the cell surface to open (e.g., G551D) or conduct Cl^- or HCO_3^- (e.g., R117H) are also found in CF patients (5, 18). Pharmacological agents that increase the flow of ions through activated CFTR present at the cell surface are called CFTR potentiators (14, 17, 19).

Here we describe the in vitro pharmacology of VX-770, the first potent and orally available CFTR potentiator to enter human clinical trials. In a Phase 2 clinical study in CF patients carrying the G551D *CFTR* mutation, oral administration of VX-770 increased CFTR activity as determined by improvement in biomarkers of CFTR function and improved lung function (20). The results shown here indicate that VX-770 increased the open probability (P_o) of the CFTR channel only after its activation by PKA and increased the CFTR-mediated transepithelial current (I_T) in cultured human bronchial epithelia (HBE) isolated from the bronchi of CF donor lungs carrying the G551D and/or F508del *CFTR* mutations. In G551D/F508del HBE, potentiation of CFTR-mediated Cl^- secretion by VX-770 reduced excessive ENaC-mediated Na^+ and fluid absorption, resulting in an increase in the surface fluid and cilia beat frequency (CBF). These results suggest that pharmacological agents that increase CFTR-mediated Cl^- secretion may improve epithelial cell function in CF.

Results

Pharmacological Characterization of VX-770. We identified VX-770 (Fig. 1*A*) from a high-throughput screening (HTS) and lead optimization effort to discover CFTR potentiators for clinical

Author contributions: F.V.G., S.H., P.D.J.G., J.W., R.A.F., and M.A. designed research; F.V.G., B.B., D.C., T.N., A.T., A.S., J.J., A.H., J.Z., J.M., V.A., C.D., J.Y., C.Y., and E.R.O. performed research; F.V.G. and B.B. analyzed data; and F.V.G. and P.N. wrote the paper.

Conflict of interest statement: F.V.G., S.H., P.D.J.G., B.B., D.C., T.N., A.T., A.S., J.J., A.H., J.Z., J.M., V.A., C.D., J.Y., C.Y., E.R.O., and P.N. are employees of Vertex Pharmaceuticals Incorporated, which is evaluating VX-770 as a potential treatment for cystic fibrosis.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0904709106/DCSupplemental.

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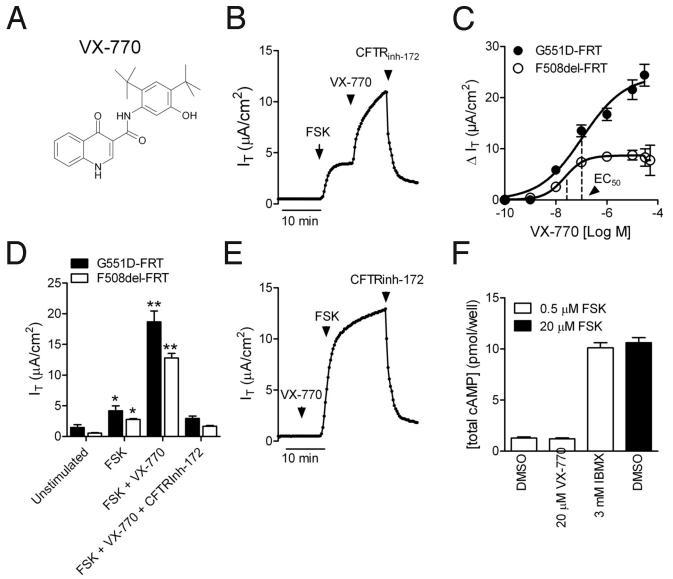

Fig. 1. VX-770 acted as a potentiator, not an activator, of G551D- and F508del CFTR in recombinant cells. (*A*) Structure of VX-770 (N-(2,4-di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide)). (*B*) Representative I_T recording from G551D-FRT showing the response to the sequential application of 10 μM forskolin (FSK), 10 μM VX-770, and 20 μM $CFTR_{inh-172}$. (*C*) Concentration–response curve of the net increase in forskolin-stimulated I_T after sequential application of VX-770 at the concentrations indicated in G551D-FRT (filled circles; $n = 12$) and temperature-corrected F508del-FRT (open circles; $n = 5$). (*D*) Mean ($\pm$ SEM) of the I_T response in G551D- (filled bars; $n = 6$) and F508del- (open bars; $n = 5$) FRT under the conditions indicated in *B*. Single asterisk indicates significant difference relative to unstimulated; double asterisk indicates significant difference relative to forskolin and unstimulated ($P < 0.05$; one-way ANOVA followed by Tukey's multiple comparison test). (*E*) Representative I_T recording from G551D-FRT showing the response to the sequential application of 10 μM VX-770, 10 μM FSK, and 20 μM $CFTR_{inh-172}$. (*F*) Total cAMP (cellular and secreted) was measured after 30 min incubation of FRT cells with 0.5 μM FSK plus DMSO, VX-770, and the nonspecific PDE inhibitor, IBMX (open bars) or with 20 μM FSK alone (filled bar).

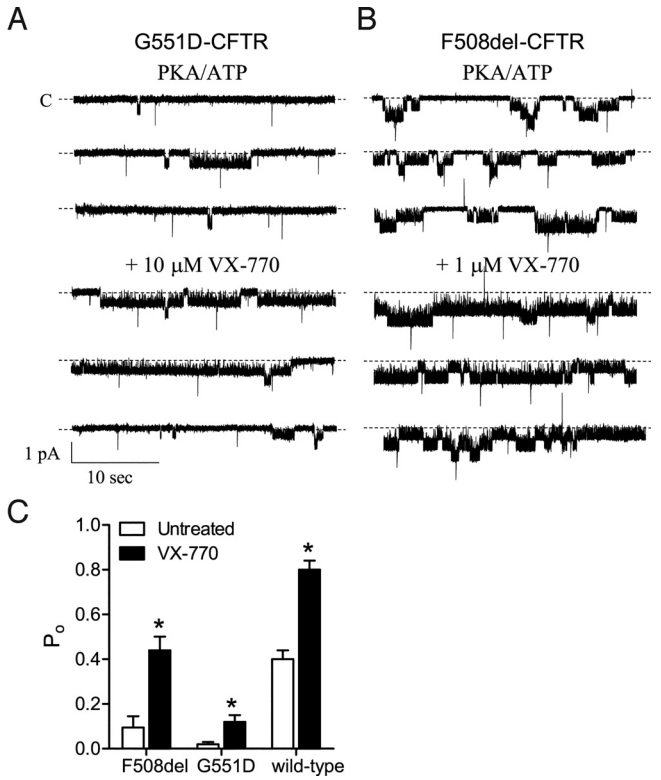

Fig. 2. VX-770 potentiated the gating activity of CFTR. Representative patch-clamp recording of the single channel current resulting from activation of G551D CFTR in FRT cells (*A*) and F508del CFTR in NIH 3T3 cells (*B*) by 1 mM ATP and 75 nM PKA before and during VX-770 application. The maximum effective concentration of VX-770 based on Fig. 1C was used for each CFTR form. All modulators were added to the cytoplasmic surface. Dotted lines indicate closed state. (*C*) Mean P_o of F508del-, G551D-, and wild-type CFTR in the presence of PKA and ATP alone (open bars) and with VX-770 (filled bars). For G551D CFTR, 10 μM VX-770 was added, whereas 1 μM VX-770 was added to F508del- and wild-type CFTR. F508del-NIH 3T3 cells were incubated at 27 °C for 24 h before recording. Asterisks indicate significant difference ($P < 0.05$; *t* test; $n = 5$–6).

development. Briefly, HTS of 228,000 chemically diverse drug-like and lead-like compounds was performed using a cell-based fluorescence membrane potential assay designed to identify CFTR potentiators (17). A lead scaffold was selected for medicinal chemistry optimization based on potency, selectivity, and chemical tractability. Several rounds of analog synthesis led to identification of VX-770. VX-770 was selected for development based on, among other criteria, its ability to potentiate multiple CFTR forms, its in vitro selectivity, and a favorable preclinical pharmacokinetic profile.

The effects of VX-770 on CFTR-mediated Cl^- secretion in vitro were assessed in both recombinant cell lines and primary cultures of HBE isolated from the bronchi of CF and non-CF donor lungs. The effect of VX-770 on CFTR-mediated Cl^- secretion was initially characterized by measuring the CFTR-mediated I_T in Ussing chambers using recombinant Fisher rat thyroid (FRT) cells expressing either human wild-type, G551D, or F508del CFTR. Consistent with the previously identified functional impairment of G551D CFTR (5), the addition of a maximally effective concentration of forskolin (10 μM) caused only a modest increase in the I_T (Fig. 1*B*) compared to wild-type CFTR-FRT cells (6.7 $\pm$ 0.9 to 50 $\pm$ 1 $\mu A/cm^2$; $n = 7$). Sequential addition of VX-770 increased the forskolin-stimulated I_T by $\approx$4-fold with an EC_{50} of 100 $\pm$ 47 nM ($n = 12$), and this response was blocked by the CFTR inhibitor $CFTR_{inh-172}$ (21) (Fig. 1 *B*–*D*). In G551D-FRT, 10 μM VX-770 alone did not increase the I_T (Fig. 1 *B* vs. *E*; unstimulated = 0.45 $\pm$ 0.03 $\mu A/cm^2$ vs. 10 μM VX-770 = 0.45 $\pm$ 0.04 $\mu A/cm^2$; $n = 3$), and VX-770 did not increase cellular cAMP levels in FRT cells (Fig. 1*F*).

To test whether VX-770 also potentiated F508del CFTR, we incubated F508del-FRT cells at 27 °C overnight to increase the cell surface density of F508del CFTR (13). VX-770 increased the forskolin-stimulated I_T in temperature-corrected F508del-FRT cells by $\approx$6-fold with an EC_{50} of 25 $\pm$ 5 nM (Fig. 1 *C* and *D*; $n = 5$). No response to forskolin and VX-770 addition was observed in FRT cells that did not express CFTR [see supporting information (SI) Fig. S1]. Taken together, the results indicate that VX-770 is a potentiator, not an activator, of G551D- and F508del CFTR–mediated Cl^- secretion.

The biophysical basis for CFTR potentiation by VX-770 was investigated by measuring the P_o of CFTR in excised membrane patches from recombinant cells expressing G551D-, F508del-, or wild-type CFTR. Before the addition of VX-770, the CFTR channel was exposed to maximally effective concentrations of PKA (75 nM) and ATP (1 mM). Under these conditions, 10 μM VX-770 increased the P_o of G551D CFTR by $\approx$6-fold (Fig. 2 *A* and *C*). VX-770 also increased the P_o of F508del- and wild-type CFTR by $\approx$5-fold and $\approx$2-fold, respectively (Fig. 2 *B* and *C*). These data are consistent with the increases in the forskolin-stimulated I_T observed with VX-770 in G551D- and F508del-FRT cells (Fig. 1), and suggest that VX-770 acts by increasing CFTR channel gating.

Effect of VX-770 in Primary Cultures of HBE Carrying G551D and F508del *CFTR* Mutations. To study the effect of VX-770 on CFTR-mediated Cl^- secretion in a more physiologically relevant cell

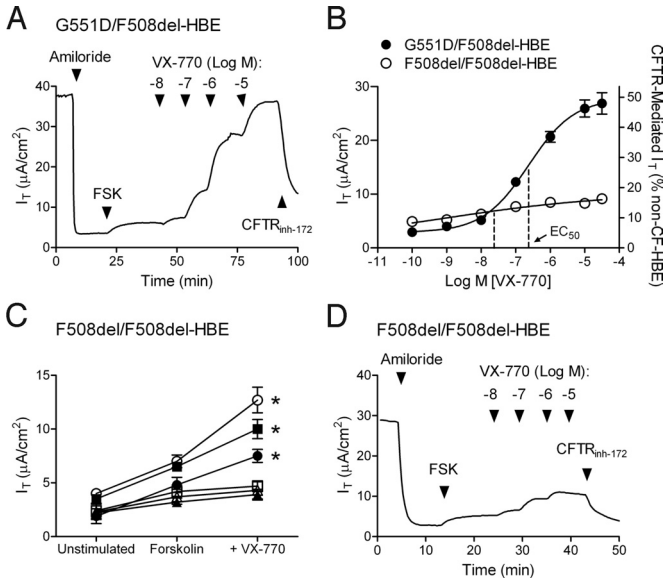

Fig. 3. VX-770 potentiated CFTR-mediated Cl^- secretion in primary cultures of G551D/F508del HBE and F508del HBE. Ussing chamber techniques were used to record the I_T resulting from CFTR-mediated Cl^- secretion. To isolate the CFTR-mediated I_T, a basolateral to apical Cl^- gradient was established, 30 μM amiloride was added to block ENaC, and 10 μM (EC_{99}) forskolin (FSK) was applied to activate CFTR. The addition $CFTR_{inh-172}$ (20 μM) to the apical surface was used to confirm that the forskolin-stimulated I_T was caused by CFTR. (*A*) Representative I_T tracing from G551D/F508del HBE. (*B*) The concentration–response curve for VX-770 in the presence of FSK is shown for G551D/F508del HBE isolated from the bronchi of a single individual (filled circles; $n = 16$) and F508del HBE isolated from the bronchi of the three individuals that responded to VX-770 (open circles; $n = 7$–24). Left *y* axis shows I_T responses; right *y* axis shows I_T normalized to the 10 μM FSK-stimulated I_T in non-CF HBE (mean ± SEM). Note that the error bars for the F508del HBE were smaller than the symbol. (*C*) The I_T before FSK addition (unstimulated) and during the sequential addition of 10 μM FSK followed by 10 μM VX-770 in F508del HBE isolated from the bronchi of six F508del-homozygous individuals ($n = 4$–24). (*D*) Representative I_T tracing from F508del HBE isolated from the bronchi represented by the open circles in *C*.

system, we used cultures of HBE isolated from the bronchi of CF donor lung tissue. Air-interface cultures of CF HBE exhibit several defects in airway epithelial function that are believed to contribute to the development of CF lung disease, including low Cl^- and fluid secretion, excessive Na^+ and fluid absorption, and decreased cilia beating secondary to decreased surface fluid (11, 17, 22). In HBE isolated from the bronchi of a single CF donor lung carrying the G551D and F508del *CFTR* mutations, VX-770 increased the forskolin-stimulated I_T with an EC_{50} of 236 ± 200 nM ($n = 16$) that was blocked by the inhibitor $CFTR_{inh-172}$ (Fig. 3 *A* and *B*). VX-770 was ≈70-fold more potent than the commonly used CFTR potentiator genistein (23), which had an EC_{50} of 16 ± 3 μM ($n = 3$). To calibrate the increase in CFTR function by VX-770, the forskolin-stimulated I_T in G551D/F508del HBE was compared to that in HBE isolated from four individuals without CF (non-CF HBE). In G551D/F508del- and non-CF HBE, the peak I_T response to 10 μM forskolin was 2.9 ± 0.5 $\mu A/cm^2$ and 56 ± 6 $\mu A/cm^2$, respectively. This indicates that the maximum forskolin-stimulated I_T in G551D/F508del HBE is equivalent to 5 ± 1% of non-CF HBE ($n = 16$), consistent with the low CFTR function and severe CF disease observed in individuals carrying both the G551D and F508del mutations (24). VX-770 increased the forskolin-stimulated I_T in G551D/F508del HBE by ≈10-fold to 27 ± 2 $\mu A/cm^2$ ($n = 16$), which is equivalent to 48 ± 4% of non-CF HBE (Fig. 3*B*). These data indicate that VX-770 is a potent and efficacious potentiator of CFTR in G551D/F508del HBE.

The effect of VX-770 on F508del CFTR was assessed in cultured HBE isolated from the bronchi of six F508del-homozygous individuals with CF (F508del HBE). In these experiments, there was no attempt to increase the cell surface density of F508del CFTR by low temperature incubation or addition of a CFTR corrector. In the absence of VX-770, a range of forskolin-stimulated I_T responses was observed with a mean increase over the unstimulated I_T of 2.2 ± 0.9 $\mu A/cm^2$ (Fig. 3*C*), equivalent to 4.0 ± 0.6% of non-CF HBE. This is consistent with the low CFTR function and severe CF disease observed in F508del-homozygous individuals (24). The addition of VX-770 caused a significant ($P < 0.05$) increase in the forskolin-stimulated I_T in F508del HBE isolated from three of the six individuals with a mean EC_{50} of 22 ± 10 nM and a maximum response of 16 ± 4% non-CF HBE (Fig. 3*B*–*D*). Thus, VX-770 was able to potentiate CFTR in some F508del HBE cultures, although the magnitude of the effect on the forskolin-stimulated I_T was less than in G551D/F508del HBE (Fig. 3*B*). Similar to the results in FRT cells, the EC_{50} in F508del HBE was lower than that in G551D/F508del HBE.

Effect of VX-770 on Na^+ and Fluid Absorption in G551D/F508del HBE. In the CF airway, the loss of CFTR function is believed to cause an increase in ENaC-mediated Na^+ absorption as evident in the elevated baseline potential difference (PD) and increased response to amiloride in nasal potential difference (NPD) recordings in CF patients (4). We monitored the baseline PD and amiloride response in cultured G551D/F508del HBE and wild-type HBE under open-circuit recording conditions that resemble the in vivo NPD assays commonly performed in individuals with CF (25). As observed in CF subjects in vivo (23), the in vitro baseline PD was elevated and the amiloride response was larger in G551D/F508del HBE compared with non-CF HBE (Fig. 4 *A* and *B*). Addition of VX-770 to G551D/F508del HBE decreased the PD with an IC_{50} of 43 ± 38 nM (Fig. 4 *B* and *C*; $n = 6$) and decreased the amiloride response (Fig. 4*D*; $n = 6$). The effect of VX-770 on the PD and amiloride response were augmented by subsequent addition of forskolin and blocked by prior addition of $CFTR_{inh-172}$ (Fig. 4 *C*–*D*), indicating that these effects were due to CFTR potentiation rather than to direct block of ENaC. This is further supported by the lack of effects of VX-770 on the isolated ENaC-mediated Na^+ current in recombinant NIH 3T3 cells expressing the rat α, β, and γ subunits of ENaC (see Fig. S2). To determine the relative contributions of CFTR-mediated Cl^- secretion and ENaC-mediated Na^+ absorption to the PD in non-CF HBE and G551D/F508del HBE, we monitored the response to amiloride and $CFTR_{inh-172}$ in the presence or absence of VX-770 with or without forskolin (Fig. 4*E*). Under these conditions, the increase in the response to $CFTR_{inh-172}$ correlated with a decrease in the amiloride sensitive PD (Fig. 4*F*). Together, these results suggest that the increase in CFTR-mediated Cl^- secretion by VX-770 caused a secondary decrease in ENaC-mediated ion transport, the latter to levels similar to those in non-CF HBE.

In the CF lung, the decrease in CFTR-mediated Cl^- and fluid secretion and the associated increase in ENaC-mediated Na^+ and fluid absorption are believed to cause the dehydration of the airway surface (9–11). To determine whether potentiation of CFTR by VX-770 was associated with an increase in the fluid levels on the apical surface of G551D/F508del HBE, we monitored the airway surface liquid (ASL) volume. To do this, we placed 100 μl of fluid on the apical surface and measured the amount remaining after up to 72 h in culture (Fig. 5*A*). To stimulate PKA-mediated activation of CFTR, 30 nM (≈EC_{99}; see Fig. S3) vasoactive intestinal peptide (VIP) was added to the basolateral surface. VIP was used because it is an abundant natural transmitter in the lung and stimulates CFTR-mediated Cl^- and fluid secretion in non-CF but not CF airway epithelium (26, 27). Although the initial rates of decline in the ASL volume were similar, the steady-state level was substantially less in G551D/F508del HBE compared with non-CF HBE (Fig. 5*A*). In G551D/F508del HBE, the addition of VX-770 increased the ASL volume to approximately half of that observed in

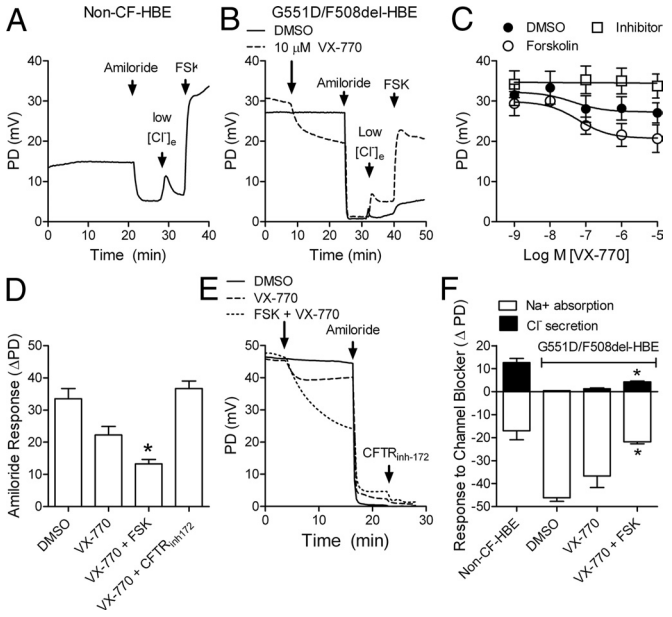

Fig. 4. VX-770 increased CFTR-mediated Cl^- secretion and reduced ENaC-mediated Na^+ absorption in G551D/F508del HBE. Representative recording of the PD in non-CF HBE (*A*) and G551D/F508del HBE (*B*) obtained with the Ussing chamber technique (open circuit mode) using equimolar Cl^- on the basolateral and apical sides. In G551D/F508del HBE, DMSO (vehicle; solid lines) or 10 μM VX-770 (dashed lines) was added to the apical surface before sequential addition of amiloride, low Cl^- (5 mM) and 10 μM forskolin (FSK) to the apical surface. (*C*) Concentration–response curve (mean ± SEM, $n = 6$) of the PD response to VX-770 addition in the presence of DMSO (filled circles), 10 μM FSK (open circles) or 20 μM $CFTR_{inh-172}$ (open squares). (*D*) Net change in PD (ΔPD) after the addition of 30 μM amiloride in the presence of the treatments indicated. (*E*) Representative PD recording in G551D/F508del HBE showing the response to DMSO (solid line) or 10 μM VX-770 without (dashed line) or with 10 μM FSK (dotted line) followed by sequential addition of 30 μM amiloride and 20 μM $CFTR_{inh-172}$. (*F*) The contribution of ENaC and CFTR to the PD in non-CF HBE and G551D/F508del HBE (bar) was determined by measuring the PD change in response to amiloride (open bars) and $CFTR_{inh-172}$ (filled bars), respectively. All data are from G551D/F508del HBE isolated from the bronchi of a single individual. Asterisks indicate significant difference compared with vehicle-treated controls (ANOVA followed by Tukey's test; mean ± SEM, $n = 6$).

non-CF HBE (Fig. 5A). The response to VX-770 was concentration dependent, blocked by $CFTR_{inh-172}$, and reduced in the absence of VIP (Fig. 5*B* and *C*). These data show that VX-770 increased the amount of fluid on the apical surface of G551D/F508del HBE, indicating less absorption, more secretion, or both.

Dehydration of the airway surface in the CF lung is believed to contribute to the inability of the cilia to extend and beat normally, preventing them from supplying the motive force for mucus transport (11). We next tested whether the increase in the apical fluid level by VX-770 in cultured G551D/F508del HBE was sufficient to increase the CBF, which was significantly reduced compared with that observed in non-CF HBE (Fig. 5 *D* and *E*). Treatment of G551D/F508del HBE with 10 μM VX-770 for 5 days increased the CBF compared with vehicle-treated controls, and this response was augmented by VIP (Fig. 5 *D* and *E*). In the presence of VIP and VX-770, the CBF in G551D/F508del HBE was similar to that observed for non-CF HBE (Fig. 5*E*). In addition, a change in the amplitude and duration of the waveform was observed, suggesting that the pattern of cilia beating was also altered by the increase in the apical fluid level (28). These results suggest that the partial restoration of Cl^- and Na^+ transport by VX-770 in G551D/F508del HBE is associated with an increase in the apical fluid level and an increase in cilia beating, and that these effects are further enhanced by augmenting CFTR activation by the addition of VIP to further stimulate the cAMP/PKA-signaling pathway.

Discussion

We demonstrated in this study that VX-770 is a potentiator of human G551D-, F508del-, and wild-type CFTR Cl^- channel function. A CFTR potentiator is a pharmacological agent that increases the flow of ions through activated CFTR channels. For a CFTR potentiator to act, CFTR must be at the cell surface, and the CFTR channel must be activated by endogenous cAMP/PKA signaling pathways such as β-adrenergic, VIP, or adenosine receptor stimulation (9, 26). Thus a potentiator such as VX-770 is expected to work in the background of the normal physiological control over CFTR function by increasing its activity only when and where it is needed. In these in vitro systems, VX-770 fulfilled this definition of a CFTR potentiator in that it increased Cl^- secretion only after stimulation of the cAMP/PKA signaling pathway (Fig. 1) and acted by increasing the P_o of the CFTR channel (Fig. 2). The ability of VX-770 to increase the P_o in excised membrane patches that are removed from the cytosolic signaling pathways suggests that VX-770 acts directly on CFTR to increase its gating activity. The direct action of VX-770 on CFTR is supported by studies indicating that VX-770 did not modulate cAMP/PKA signaling (Fig. 1*F*). Further studies are required to define the molecular mechanism by which VX-770 potentiates CFTR and to determine whether VX-770 also acts on other defective CFTR forms with other *CFTR* mutations. Additional studies are also required to determine whether VX-770 potentiates CFTR-mediated HCO_3^- secretion, a defect that is believed to contribute to abnormal epithelial function in some organs affected in CF, including the lung and pancreas (29).

In vivo studies correlating CFTR activity in CF patients with disease severity suggest that, as a group, individuals exhibiting 10–25% of the CFTR activity observed in non-CF individuals have less severe disease as assessed by age of diagnosis, pulmonary function, and pancreatic function than individuals with no detectible CFTR activity (14, 30–33). The results reported here show that VX-770 increased CFTR-mediated Cl^- secretion in G551D/F508del HBE from ≈5% to a maximum level of ≈50% of that measured in non-CF HBE. Based on these in vitro studies, VX-770 might be expected to result in clinical benefit in CF patients carrying the G551D mutation on at least one allele.

VX-770 also increased CFTR-mediated Cl^- secretion in cultured HBE isolated from the bronchi of some F508del-homozygous CF individuals to levels above 10% of that observed in non-CF HBE. The reason for the variable CFTR function in cultured HBE derived from different F508del-homozygous donor lungs is not known, but could be due to polyvariant mutant *CFTR* genes, such as the M470 variant (34), or other genes that affect CFTR levels or activity. Nevertheless, this provocative result suggests that the efficacy of VX-770 in G551D/F508del HBE may be caused by potentiation of both G551D- and F508del CFTR and that consideration should be given to testing VX-770 in F508del-homozygous CF patients. This observation also supports a rationale for assessing the effects of VX-770 in combination with CFTR correctors that are designed to increase the cell surface density of F508del CFTR (17).

In both FRT cells and HBE, the EC_{50} for VX-770 was lower for F508del CFTR than for G551D CFTR, suggesting that VX-770 may have a higher affinity for F508del CFTR. Other CFTR potentiators, including genistein, are also more potent against F508del CFTR compared with G551D CFTR (19). The lower potency of some CFTR potentiators against G551D CFTR along with homology modeling studies have suggested that these compounds bind at a site near the G551D mutation (35). The ability of VX-770 to potentiate CFTR in some F508del HBE is consistent with previous studies showing the presence of low levels of F508del CFTR function in vitro (17) and in vivo (36).

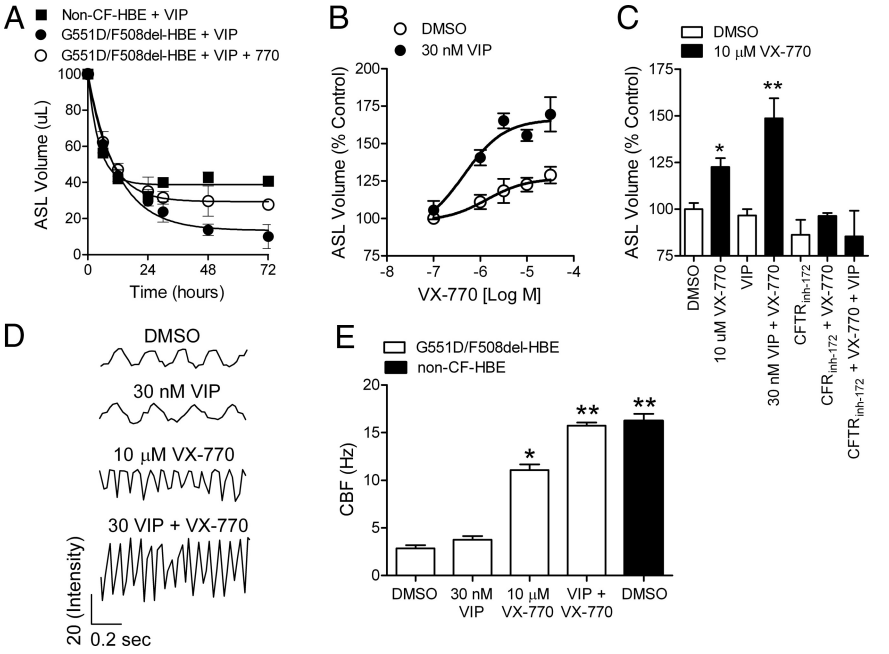

Fig. 5. Potentiation of CFTR by VX-770 partially restored fluid regulation and cilia beating in G551D/F508del HBE. (*A*) The ASL volume in G551D/F508del HBE and wild-type HBE after up to 72 hours' incubation at 37 °C in the presence of 30 nM VIP with or without VX-770. All modulators were added to the basolateral surface. (*B*) Concentration–response curve (n = 3–9) of the change in ASL volume in G551D/F508del HBE after VX-770 addition at the indicated concentrations in the absence (open circles) or presence (filled circles) of 30 nM VIP. All data were normalized to ASL volume in the vehicle (DMSO)–treated controls and expressed as percent control. (*C*) Mean (n = 3–9) ASL volume in the absence (open bars) or presence (filled bars) of 10 μM VX-770 and in the presence of 30 nM VIP and/or 20 μM $CFTR_{inh\text{-}172}$. (*D*) Representative tracings of the light intensity (y axis in relative units) derived from a single region of interest in G551D/F508del HBE monitored 5 days after adding 100 μl of fluid to the apical surface. (*E*) Mean (± SEM; n = 6) CBF wild-type HBE (filled bars) or G551D/F508del HBE (open bars) after a 5-day treatment with DMSO, 30 nM VIP, 10 μM VX-770, or 30 nM VIP with 10 μM VX-770. Single asterisk indicates significantly different ($P < 0.05$) from vehicle control in G551D/F508del HBE; double asterisk indicates significantly different ($P < 0.05$) from vehicle control and VX-770 alone. All data are from G551D/F508del HBE isolated from the bronchi of a single individual.

A long-standing question in the CF field is whether pharmacological agents that directly target and restore the loss in CFTR-mediated Cl^- secretion would be sufficient to slow or stop the deleterious cascade of events that lead to disease progression in CF patients, including dehydration of the airway surface and the inability of the airway to clear mucus and microbes (9, 11, 22). In this study, open-circuit recordings using cultured G551D/F508del HBE and non-CF HBE were performed in a way that resembles the in vivo assays of nasal PD that are commonly performed in CF patients (25). As observed in vivo, the baseline PD was elevated and the amiloride response was larger in G551D/F508del HBE compared with that in non-CF HBE. This has been attributed, at least in part, to excessive ENaC-mediated Na^+ absorption in CF tissue compared with non-CF tissue (4, 25, 37, 38). Addition of VX-770 decreased the baseline PD and amiloride response in G551D/F508del HBE. Although these PD results do not rule out the possibility that VX-770 may have effects on other channels (39) or proteins, the data may be explained by CFTR potentiation leading to a secondary decrease in ENaC activity. This is supported by the lack of direct inhibition of the rat α-, β-, γ-ENaC channel; however, differences between species and/or subunit stoichiometry between the recombinant and HBE culture models may alter the activity of VX-770 on the isolated human ENaC. In addition, other studies have shown that pharmacological or gene-transfer correction of CFTR function in CF airway cell cultures restored the balance between Cl^- secretion and Na^+ absorption (40, 41).

Several hypotheses have been proposed to explain the link between the loss in CFTR function and the increase in ENaC function in the CF airway, including changes in cytoplasmic pH (42) and Cl^- concentration (43), physical coupling between the two proteins (44), and activation of ENaC by airway surface fluid proteases (45). Our results are consistent with a model proposed by Horisberger (46) whereby the increase in CFTR-mediated Cl^- secretion caused by VX-770 would be expected to depolarize the apical membrane and thereby reduce the electrochemical driving force for Na^+ absorption through ENaC coexpressed along with CFTR in the apical membrane. This is supported by data suggesting the acute effects of VX-770 on Na^+ absorption, the ability of a CFTR inhibitor to block this effect, and the lack of direct ENaC inhibition by VX-770 in cells that do not express CFTR.

The increase in the fluid level on the apical surface of G551D/F508del HBE after several hours of treatment with VX-770 can be explained by an increase in CFTR-mediated Cl^- secretion and the concomitant decrease in excessive ENaC-mediated Na^+ absorption. Although the amiloride response after addition of VX-770 to G551D/F508del HBE was similar to that in non-CF HBE, the apical fluid level at steady-state was approximately half of that observed in non-CF HBE (Fig. 5*A*). This may be caused by the incomplete restoration of CFTR-dependent fluid secretory function in these cultures of ciliated airway epithelium. Nevertheless, the increase in apical fluid levels in VX-770–treated G551D/F508del HBE was sufficient to increase cilia beating to levels observed in non-CF HBE. In vivo, we hypothesize that the increase in height of the apical fluid level and frequency of cilia beating would facilitate the mechanical clearance of mucus and microbes, the failure of which is thought to contribute to CF lung pathogenesis (9).

Although VX-770 had some effect on unstimulated HBE, likely because of endogenous levels of cAMP in these cells (47), the maximal pharmacological effect of VX-770 on ion and fluid transport required stimulation of the cAMP/PKA signaling pathway by either forskolin or VIP. It is likely that the situation is more dynamic in vivo, where neuronal pathways and mechanical forces exerted on the airway regulate the epithelial cellular signaling pathways to control the activity of CFTR and other ion transport pathways to regulate fluid transport (48, 49). Because of this, it is possible that the endogenous level of CFTR activation will be higher in vivo. However, the ability of cAMP agonists to augment the effects of VX-770 in vitro raises the question of whether pharmacological activation of CFTR by agents that stimulate the cAMP/PKA-signaling pathway in vivo, such as the β-adrenergic agonists, would enhance any potential clinical benefit of VX-770.

Although transgenic mouse models of CF carrying the G551D or F508del mutation have been developed, we did not test VX-770 in these models because it does not potentiate mouse CFTR expressed in FRT cells (Fig. S4). This difference, however, may be exploited to understand the mechanism of action and binding site of VX-770. Also, because the cell background is the same as that used for expression of the human CFTR forms, these results support the proposed direct action of VX-770 on CFTR.

In conclusion, these in vitro studies demonstrated that VX-770 is a CFTR potentiator that increased CFTR-mediated Cl^- transport

by increasing the P_o of activated CFTR. In G551D/F508del HBE, the increase in CFTR-mediated Cl^- secretion by VX-770 was associated with a decrease in amiloride-sensitive Na^+ absorption and an increase in the apical fluid height and CBF. The results of these in vitro experiments support the hypothesis that drugs aimed at increasing CFTR function may ameliorate the downstream physiological processes that contribute to CF lung disease.

Materials and Methods

Recombinant and Human Bronchial Epithelial Cell Culture. FRT cells expressing human G551D CFTR or mouse wild-type CFTR and NIH 3T3 cells expressing human wild-type-, human F508del CFTR or the rat α, β, and γ subunits of ENaC were cultured as previously described (17). HBE were isolated from the bronchi of lungs obtained from non-CF or CF individuals after autopsy or lung transplantation and cultured as previously described (17). For details, see SI Materials and Methods.

Ussing Chamber Recordings. Ussing chamber techniques using cultured FRT and HBE cells were used to record the I_T in the voltage-clamp mode (V_{hold} = 0 mV) or the PD (PD = $V_{basolateral} - V_{apical}$) in the open-circuit recording mode (see SI Materials and Methods).

cAMP Measurements. The total cAMP concentration in FRT cells following test compound application was determined using a cAMP-Screen 96-Well Immunoassay System (see SI Materials and Methods).

Patch-Clamp Recordings. The single-channel activity of G551D CFTR, wild-type CFTR, and temperature-corrected F508del CFTR was measured using excised inside-out membrane patch recordings as previously described (17). The isolated Na^+ current in ENaC-NIH 3T3 cells was recorded using the whole-cell patch clamp recording configuration (for details, see SI Materials and Methods).

Measurement of ASL Volume and CBF. To monitor changes in the ASL volume and CBF, the apical layer of G551D/F508del HBE was washed twice with absorption buffer followed by addition of 100 μl of absorption buffer to the apical layer. To monitor the ASL volume, the fluid remaining after up to 72 h of incubation with test compounds was aspirated from the apical surface and placed in preweighed tubes. The CBF was monitored as previously described (50) (for details, see SI Materials and Methods).

Statistical Analyses. Statistical comparisons were made using analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t* test. All data are presented as the mean ± SEM.

ACKNOWLEDGMENTS. We thank Cystic Fibrosis Foundation Therapeutics Inc. (CFFT) for their generous financial support, as well as Robert Beall, Preston Campbell, John Chabala, Eric Gordon, Diana Wetmore, and Christopher M. Penland for their guidance. We also thank Dr. Joseph W. Pilewski for providing G551D/F508del HBE, Dr. L. J. V. Galietta for providing the G551D-FRT cells, Dr. Michael J. Welsh for providing the NIH 3T3 cells expressing F508del- and wild-type CFTR, and Dr. Mary Lang-Furr for providing the NIH 3T3 cells expressing ENaC. Vertex Pharmaceuticals and CFFT have a financial interest in the discovery and development of therapies for CF.

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